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Mutation Induction by DBD Plasma in Phosphate-solubilizing Bacteria *Enterobacter Agglomerans*

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Abstract

An efficient agar plate mutagenesis and screening technique for improving mutation frequency was established by using dielectric barrier discharge (DBD) plasma. *Enterobacter agglomerans* is well known as a phosphate-solubilizing plant-associated bacterium. In this study, DBD plasma was conducted toE. *agglomerans* mutation for improving the phosphate-soubilizing activity. The results showed that the phosphate-soubilizing activity of mutants increased compared with original strain, and the phosphate-soubilizing activity of the best mutants is 1.49-fold of original strain. It demonstrated that DBD plasma treatment has a high-efficient quality, and it will be a useful method of mutation.

Keywords: DBD plasma mutation；Phosphate-solubilizing；Enterobacter agglomerans

1. Introduction

Nowadays, cold atmospheric plasma treatment of microorganisms and living tissues has become a popular topic in modern plasma physics and in medical science. The plasma is capable of bacterial inactivation and non-inflammatory tissue modification, which makes it an attractive tool for treatment of

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skin disease, open injuries and dental caries. Dielectric barrier discharge(DBD), with its advantages of operating at atmospheric or medium pressure, free of arc discharge even at reasonably high power levels, has been widely investigated for some emerging applications such as biological and chemical decontamination of media at ambient conditions[1-3]. The DBD plasma is created between two conductive electrodes connected to an ac or pulsed power supply. At least one of the electrodes is covered by dielectric layer, which prevents the arc formation after breakdown. Though the high breakdown voltage in gasses at atmospheric pressure(several kv), the average electric current is low. Therefore, a DBD with its advantages of fast, low temperature, easy to operate, non-toxic and inexpensive, can be applied in direct contact with living tissues and open injuries without causing any damage[4]. The biological effects of DBD plasma are assumed to be caused by several factors as active radicals(mainly those involving oxygen), accelerated ions and UV radiation during the DBD treatment[5]. The bacterial sterilization and the effect on some microorganisms by a DBD in air have been studied and discussed, the results showed that DBD was able to induce bactericidal effects in both Gram-positive and Gram-negative microorganisms by different treatment times[5; 3]. However, few studies of microorganisms mutation using DBD treatment have been reported.

In this study, air DBD plasma at atmospheric pressure is employed to mutate phosphate-solubilizing bacteria (PSB) *Enterobacter agglomerans*. PSB can transform the insoluble phosphorus (P) to soluble forms HPO_4^{2-} and $H_2PO_4^-$ by acidification, chelation, exchange reactions, and polymeric substances formation. P, next to nitrogen, is the second important macronutrient required for plant growth. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems as well. Usually the soils are supplemented with inorganic P in the form of chemical fertilizers. A large proportion of the applied P gets fixed in the soil as phosphates of iron, aluminum and calcium[6]. This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable[7]. The use of PSB in agricultural practice would not only offset the high cost of anufacturing phosphatic fertilizers but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied[8].

The main objectives of the current study were to (i) conduct air DBD plasma for PSB E. *agglomerans* mutation to improve the phosphate-soubilizing activity; (ii) examine the changes of E. *agglomerans* in order to evaluate the efficiency of DBD plasma mutation method.

2. Materials and methods

2.1. Plasma discharge

The experimental arrangement used for DBD plasma mutation is shown in Fig.1. The operational parameters were as follows: voltage is 30 V; electric current was 0.5A. Different mutagenesis time 0, 1, 2, 3, 4, 6 and 8 min was set in order to get the optimum time that lethality precentage could up to 90% to improve the mutation efficiency.

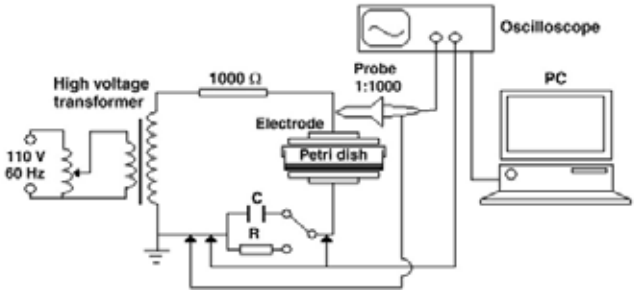

Fig. 1. Experimental equipment

2.2. Strains and culture conditions

E. agglomerans K-7 (from our lab) were isolated using conventional methodology and identified using 16S rRNA gene sequence analysis by the Beijing Sunbiotech Co., Ltd, Beijing, China. It was maintained at -80℃ in peptone water medium containing 20% glycerol.

2.3. DBD plasma mutation

E. agglomerans K-7 inoculated in LB (peptone 10 g; yeast extract 5 g; NaCl 5 g; distilled water 1000 mL; pH 7.0) medium and incubated at 28℃ on shaker at speed of 120 rpm for 12 h. Then the suspensions of PSB was standardized to absorption value of 1 at 600 nm by optical spectrophotometer. One mL bacterial suspension was transferred to a diameter 9 cm petri dish, then put the petri dish on one electrode, and kept the distance between the upper electrode and petri dish surface with 3 mm. After treatment, transferred the liquid in petri dish to phosphate-solubilizing agar plate to screen strains with higer phosphate-solubilizing ability.

2.4. Phosphorus solubilization

Solubilization of P by *E. agglomerans* K-7 after DBD plasma mutation was estimated using insoluble $Ca_3(PO_4)_2$ in National Botanical Research Institute's Phosphate (NBRIP) broth medium[9]. The composition of the medium was (g l^{-1}): glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2 \cdot 6H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 0.25 ; KCl, 0.2; $(NH_4)_2SO_4$, 0.1. One mL bacterial suspension was transferred to a 250 mL Erlenmeyer flask containing 80 mL of the NBRIP broth medium and incubated for 7 days. A separate NBRIP broth medium inoculated with sterile Milli-Q water served as the control treatment. The cultures were harvested by centrifugation at 10174 g for 10 min and soluble-P content of culture supernatant was estimated by the phosphomolybdate method[10]. The pH was determined with pH meter.

3. Results and discussion

3.1. Lethality test

Calculated the lethality of E. agglomerans K-7 based on the suvival rate in phosphate-solubilizing agar plate, and the results showed in Fig.2. In the first three minute, it was observed that the lethality

increased steeply, then it gradually to a stable level and the lethality was about 90%. In the following experiments, 3 min was choosed as the mutagenesis time.

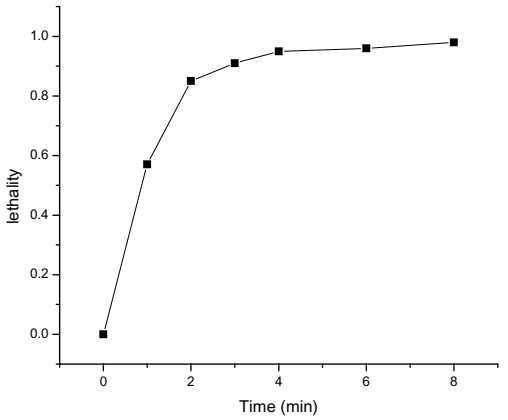

Fig. 2. Lethality of *E. agglomerans* K-7 on agar plates by DBD plasma inducement

3.2. Mutation screening

The test of the relative efficiency of isolated strains was carried out by selecting the microorganisms which are capable of producing a halo/clear zone on plate due to the production of organic acids into the surrounding medium[11]. After induced by DBD plasma, isolates with big transparent zone were pick up for further screening(see Fig.3.). In this study, 16 mutants were picked out and the halo and colony diameters were measured after 14 days of the incubation of plates at 28 ℃. The results showed in Table 1. Five mutants were choosed out and they are K-74, K-77, K-710, K-711 and K-712, respectively.

Fig. 3. Transparent zone of *E. agglomerans* K-7 induced by DBD plasma

Table 1. Colony diameter and halo zone diameter and their ratio in NBRIP medium

No.	Strains	Colony diameter(d) (cm)	Halo zone diameter(D) (cm)	D/d
1	K-7(original strain)	0.21	0.58	**2.76**
2	K-71	0.13	0.37	2.85
3	K-72	0.16	0.42	2.63
4	K-73	0.19	0.53	2.77
5	**K-74**	0.11	0.33	**3.01**
6	K-75	0.20	0.57	2.84
7	K-76	0.22	0.61	2.79
8	**K-77**	0.18	0.52	**2.89**
9	K-78	0.16	0.44	2.75
10	K-79	0.16	0.43	2.68
11	**K-710**	0.15	0.47	**3.12**
12	**K-711**	0.13	0.37	**2.88**
13	**K-712**	0.13	0.38	**2.96**
14	K-713	0.15	0.43	2.84
15	K-714	0.13	0.36	2.75
16	K-715	0.12	0.32	2.63
17	K-716	0.15	0.41	2.76

3.3. Phosphate-solubilizing ability test

Quantitative estimation of phosphate solubilization in broth was carried out using the five strains K-74, K-77, K-710, K-711 and K-712 screened above. The results showed in Table 2. It was observed that the pH is lower when inoculated mutant strains compared to inoculated original strain K-7 in the solution, and what with it correspondence was that the ability of solubilizing phosphate is better too. According to the experiments for screening phosphate solubilizing isolates, a higher soluble phosphate production always combined with a lower pH (data not shown). A inverse relationship between pH and soluble phosphate has been reported before[12-13]. It indicated that phosphate solubility was directly correlated with the acids produced. There were reports that PSB released many kinds of organic acids, such as oxialic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glyconic, fumaric, adipic, and 2-ketogluconic acids[14-15]. It was interesting to note that the amount of organic acid the mutants excreted increased after induced by DBD plasma. In comparison to the original strain K-7, the ability of phosphate-solubilizing of mutant K-710 is increased 1.49-fold.

Table 2. pH value and soluble phosphate in NBRIP medium after 7 days

Strains	pH	Solubilizing phosphate (mg/L)
K-7(original strain)	4.3	320.1
K-74	3.8	456.2
K-77	4.1	372.4
K-710	3.9	**475.9**
K-711	4.1	381.3
K-712	4.0	398.4

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